04-14-04

TRANSMITTAL LETTER (General - Patent Pending)	Docket No. TSR-10002/38

In Re Application Of: **John Hilfinger et al.**

Serial No.	Filing Date	Examiner	Group Art Unit
10/706,738	November 12, 2003		

Title: **METHODS AND COMPOSITIONS OF GEGNE DELIVERY AGGENTS FOR SYSTEMIC AND LOCAL THERAPY**

TO THE COMMISSIONER FOR PATENTS:

Transmitted herewith is:

1. **Petition to Obtain Filing Date**
2. **Petition Fee of $130**

in the above identified application.

- ☐ No additional fee is required.
- ☒ A check in the amount of **$130.00** is attached.
- ☒ The Director is hereby authorized to charge and credit Deposit Account No. **07-1180** as described below.
  - ☐ Charge the amount of
  - ☒ Credit any overpayment.
  - ☒ Charge any additional fee required.

Avery N. Goldstein

Signature

Dated: April 13, 2004

Avery N. Goldstein, Reg. No. 39,204
Attorney for Applicant
Gifford, Krass, Groh, Sprinkle,
Anderson & Citkowski, P.C.
280 N. Old Woodward Avenue, Suite400
Birmingham, MI 48009-5394
(248) 647-6000

EV435301293US

I certify that this document and fee is being deposited on April 13, 2004 with the U.S. Postal Service as ~~first class mail~~ under 37 C.F.R. 1.8 and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450. EXPRESS MAIL

Janice R. Kuehn

Signature of Person Mailing Correspondence

Janice R. Kuehn

Typed or Printed Name of Person Mailing Correspondence

cc:

Attorney Docket No. TSR-10002/38

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Hilfinger et al.

Serial No.: 10/706,738

Filing Date: November 12, 2003

For: METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

PETITION TO OBTAIN FILING DATE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In accordance with 37 CFR Section 1.53(b), Applicant hereby requests grant of the filing date of November 12, 2003 and the inclusion of the misplaced pages.

Statement of Facts

On November 12, 2003 Applicant filed a utility patent application that is a non-provisional of U.S. Provisional Patent Application No. 60/425,379, filed November 12, 2002. The utility patent application was assigned Serial No. 10/706,738. The utility patent application incorporated by reference the text of U.S. Provisional Patent Application No. 60/425,379. (See page 1 of specification).

The entire patent application transmittal as originally filed is included herewith at Exhibit A.

As part of the utility patent application submission a return receipt postcard was provided (transmittal documents, page 2 of 4, item 14). A copy of the return receipt postcard is attached hereto as Exhibit B. The return receipt postcard indicated that the five pages of claims were not received as part of the submission package. A telephone call to OIPE resulted in the recommendation that we delay any inquiry until after image file wrapper capture had been completed.

On February 25, 2004, a paralegal in our office, Janice Kuehn, who had initially worked to prepare the transmittal for the above-referenced utility patent application, entered the Patent Application Information Retrieval system (PAIR) using our customer number and identified not only the omission of five pages of claims according to the return receipt postcard, but also that the specification noted on the postcard as having 27 pages numbered only 12 pages upon image file wrapper capture. Subsequent phone calls were made to Ms. Dalord Dillard and Mr. Kevin Diggs of OIPE in order to request recovery of the original mailing material corresponding to this case in order to locate the missing specification and/or claims pages. These telephone communications attempting to gain additional information from Ms. Dillard and Mr. Diggs have continued from late February 2004 through until April 5, 2004. As of April 5, 2004, no information has been made available to Applicant by OIPE with respect to the content of the original mailing materials.

In light of the acknowledgement provided in the postcard (Exhibit B) indicating the acceptance of 27 pages of specification, Applicant hereby petitions to have the missing 15 pages of specification (pages numbered 13-27 inclusive) restored to the application file.

The declaration of Janice R. Kuehn attached as Exhibit C details the procedure by which the application submission was prepared and is provided to evidence a procedure that makes the

omission from the utility patent application submission packet of fifteen pages of specification or five pages of claims highly unlikely.

As a separate basis for the granting of the filing date of November 12, 2003 to the above-referenced utility patent application is the fact that U.S. Provisional Patent Application Serial No. 60/425,379 was incorporated by reference. The full text of Provisional Patent Application Serial No. 60/425,379 is found at appended Exhibit D. As the instant utility application incorporates by reference this provisional application which itself had patent claims appended thereto, it is submitted that this represents an independent basis for the grant of the original filing date and the subsequent filing of a preliminary amendment to include the missing five pages of claims.

Petition Fee

Provided herewith is a petition fee under 37 CFR Section 1.17(i). Request for a refund of the petition fee is requested if it is determined that the pages of specification numbered 13-27 inclusive and claim pages numbered 28-32 inclusive (corresponding to claims 1-25) are subsequently determined to in fact have been received by the PTO along with the evidence provided herein of such deposit.

Insertion of specification pages 13-27 inclusive of the specification is requested. Acceptance of the missing five pages of claims corresponding to claims 1-25, or an

acknowledgement that through the incorporation of the provisional application including claims that the present application will be accorded a filing date of November 12, 2003 is requested.

Respectfully submitted,

Avery N. Goldstein

Avery N. Goldstein
Registration No. 39,204
Gifford, Krass, Groh, Sprinkle,
Anderson & Citkowski, P.C.
280 N. Old Woodward Ave., Suite 400
Birmingham, MI 48009-5394
(248) 647-6000

Attorney for Applicant

ANG/gs
Enclosures

GS–W:\Word Processing\ang\TSR10002-PetitionForFilingDate.doc

CERTIFICATE OF MAILING BY "EXPRESS MAIL"

"EXPRESS MAIL" MAILING LABEL NUMBER EV435301293US

DATE OF DEPOSIT April 13, 2004

I hereby certify that this paper or fee (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office To Addressee" Service under 37 CFR 1.10 on the date indicated above and is addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Janice R. Kuehn

Janice R. Kuehn

Express Mail Label No. EL 983172102 US

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. TSR-10002/38
Total Pages in this Submission 52

TO THE COMMISSIONER FOR PATENTS
Mail Stop Patent Application
P.O. Box 1450
Alexandria, VA 22313-1450

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

and invented by:

John Hilfinger, Blake Roessler, and Phillip Kish

If a CONTINUATION APPLICATION, *check appropriate box and supply the requisite information:*

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Which is a:

☐ **Continuation** ☐ **Divisional** ☐ **Continuation-in-part (CIP)** of prior application No.: _____

Enclosed are:

Application Elements

1. ☒ Filing fee as calculated and transmitted as described below
2. ☒ Specification having 34 pages and including the following:
   - a. ☒ Descriptive Title of the Invention
   - b. ☒ Cross References to Related Applications *(if applicable)*
   - c. ☐ Statement Regarding Federally-sponsored Research/Development *(if applicable)*
   - d. ☐ Reference to Sequence Listing, a Table, or a Computer Program Listing Appendix
   - e. ☒ Background of the Invention
   - f. ☒ Brief Summary of the Invention
   - g. ☒ Brief Description of the Drawings *(if filed)*
   - h. ☒ Detailed Description
   - i. ☒ Claim(s) as Classified Below
   - j. ☒ Abstract of the Disclosure

EL983172102US

25006
PATENT TRADEMARK OFFICE

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No. TSR-10002/38
Total Pages in this Submission 52

Application Elements (Continued)

3. ☒ Drawing(s) *(when necessary as prescribed by 35 USC 113)*
   a. ☒ Formal Number of Sheets 7
   b. ☐ Informal Number of Sheets _____
4. ☒ Oath or Declaration
   a. ☒ Newly executed *(original or copy)* ☐ Unexecuted
   b. ☐ Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional application only)*
   c. ☒ With Power of Attorney ☐ Without Power of Attorney
   d. ☐ *DELETION OF INVENTOR(S)*
      Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. ☐ Incorporation By Reference *(usable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
6. ☐ CD ROM or CD-R in duplicate, large table or Computer Program (Appendix)
7. ☒ Application Data Sheet (See 37 CFR 1.76)
8. ☐ Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all must be included)*
   a. ☐ Computer Readable Form (CFR)
   b. ☐ Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statement(s) Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 CFR 3.73(B) Statement (when there is an assignee)
11. ☐ English Translation Document (if applicable)
12. ☐ Information Disclosure Statement/PTO-1449 ☐ Copies of IDS Citations
13. ☐ Preliminary Amendment
14. ☒ Return Receipt Postcard (MPEP 503) (Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
16. ☒ Certificate of Mailing

    ☐ First Class ☒ Express Mail *(Specify Label No.):* EL 983172102 US

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
TSR-10002/38
Total Pages in this Submission
52

Accompanying Application Parts (Continued)

17. ☒ Applicant claims small entity status. See 37 CFR 1.27.

    ☐ (Optional) Small Entity Statement(s) - Specify Number of Statements Submitted: _____

18. ☐ Additional Enclosures *(please identify below):*

Request That Application Not Be Published Pursuant To 35 U.S.C. 122(b)(2)

19. ☐ Pursuant to 35 U.S.C. 122(b)(2), Applicant hereby requests that this patent application not be published pursuant to 35 U.S.C. 122(b)(1). Applicant hereby certifies that the invention disclosed in this application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication of applications 18 months after filing of the application.

Warning

An applicant who makes a request not to publish, but who subsequently files in a foreign country or under a multilateral international agreement specified in 35 U.S.C. 122(b)(2)(B)(i), must notify the Director of such filing not later than 45 days after the date of the filing of such foreign or international application. A failure of the applicant to provide such notice within the prescribed period shall result in the application being regarded as abandoned, unless it is shown to the satisfaction of the Director that the delay in submitting the notice was unintentional.

UTILITY PATENT APPLICATION TRANSMITTAL
(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
TSR-10002/38
Total Pages in this Submission
52

Fee Calculation and Transmittal

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	25	- 20 =	5	x $9.00	$45.00
Indep. Claims	4	- 3 =	1	x $43.00	$43.00
Multiple Dependent Claims (check if applicable) ☐					$0.00
				BASIC FEE	$385.00
OTHER FEE *(specify purpose)* _____					$0.00
				TOTAL FILING FEE	$473.00

☒ A check in the amount of $473.00 to cover the filing fee is enclosed.

☐ The Director is hereby authorized to charge and credit Deposit Account No. 07-1180 as described below.

- ☐ Charge the amount of as filing fee.
- ☒ Credit any overpayment.
- ☒ Charge any additional filing fees required under 37 C.F.R. 1.16 and 1.17.
- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: November 11, 2005

Signature

Avery N. Goldstein, Reg. No. 39,204
Attorney for Applicant
Gifford, Krass, Groh, Sprinkle,
Anderson & Citkowski, P.C.
280 N. Old Woodward Avenue, Suite 400
Birmingham, MI 48009-5394
(248) 647-6000

cc:

APPLICATION DATA SHEET

Electronic Version v14
Stylesheet Version v14.0

Title of Invention	METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

Application Type: regular, utility
Attorney Docket Number: TSR-10002/38

Correspondence address:
Customer Number: 25006 *25006*

Continuing Data:

This is a Non-Provisional of US application number 60/425,379, filed 2002-11-12 , now pending.

Inventors Information:

Inventor 1:

Applicant Authority Type:	Inventor
Citizenship:	US
Given Name:	John
Family Name:	Hilfinger
City of Residence:	Ann Arbor
State of Residence:	MI
Country of Residence:	US
Address-1 of Mailing Address:	2636 Essex
Address-2 of Mailing Address:	
City of Mailing Address:	Ann Arbor
State of Mailing Address:	MI
Postal Code of Mailing Address:	48104
Country of Mailing Address:	US
Phone:	
Fax:	
E-mail:	

Inventor 2:

Applicant Authority Type:	Inventor
Citizenship:	US
Given Name:	Blake
Family Name:	Roessler
City of Residence:	Ann Arbor
State of Residence:	MI
Country of Residence:	US
Address-1 of Mailing Address:	3175 Dolph Drive
Address-2 of Mailing Address:	
City of Mailing Address:	Ann Arbor
State of Mailing Address:	MI
Postal Code of Mailing Address:	48103
Country of Mailing Address:	US
Phone:	
Fax:	
E-mail:	

Inventor 3:

Applicant Authority Type:	Inventor
Citizenship:	US
Given Name:	Phillip
Family Name:	Kish
City of Residence:	Ann Arbor
State of Residence:	MI
Country of Residence:	US
Address-1 of Mailing Address:	4785 Lake Court
Address-2 of Mailing Address:	
City of Mailing Address:	Ann Arbor
State of Mailing Address:	MI
Postal Code of Mailing Address:	48103
Country of Mailing Address:	US
Phone:	
Fax:	

E-mail:

Attorney Information:

practitioner(s) at Customer Number:

25006 *25006*

as our attorney(s) or agent(s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

Publication Information:
Suggested Figure for Publication -
Suggested Classification -
Suggested Technology Center -
Total Number of Drawing Sheets - 7

TSR-10002/38
31105gs

METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

RELATED APPLICATION

This application claims priority of United States Provisional Patent Application Serial No. 60/425,379 filed November 12, 2002, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to gene therapy and more particularly to drug delivery molecules for delivering various nucleic acid compositions to the lumen of the intestine or to localized tissues.

BACKGROUND OF THE INVENTION

Gene therapy has garnered considerable attention as a method to treat various human diseases by the enhancement of protein production. These include gene replacement or gene augmentation.

The delivery of genetic material into a multi-celled organism has proven more difficult than initially imagined. A variety of techniques have been developed to accomplish *in vivo* transformation of cells including direct injection of nucleic acid or a particle decorated with nucleic acid directly into cells, recombinant viruses, liposomes and receptor mediated endocytosis.

In attempting to develop lower cost modes of administration that are likely to enhance patient compliance, intestinal gene therapy has been recognized as an attractive situs for *in vivo* gene therapy owing to the ease of

access through oral or rectal routes. However, the intestine routinely degrades large quantities of foreign nucleic acid ingested as part of foodstuffs. DNAses and RNAses in the intestinal tract represent a significant barrier to the entry of intact and functional nucleic acids to intestinal tract cells.

U.S. Patent 6,225,290 represents an effort to deliver bare nucleic acid sequences through laparotomy, oral or suppository administration but is silent as to nucleic acid protection and overcoming the above-stated problems of intestinal administration. It is generally agreed that an oral administration of nucleic acids represents the least expensive and most likely route for the attainment of patient compliance with dosing requirements.

While oral administration is generally recognized as the superior route, little attention has been paid to methodologies and packaging of DNA to preclude intestinal degradation. U.S. Patent 6,500,807 is representative of an attempt to produce a protective coating of carbohydrate around a nucleic acid to facilitate oral administration. It would be advantageous to deliver nucleic acids in a form other than micelles to facilitate conventional pharmaceutical compounding. Thus, there exists a need for gene delivery agents amenable to efficient transfection of target cells capable of inducing systemic and/or local transfection.

SUMMARY OF THE INVENTION

A method is provided for the packaging of a nucleic acid with a chelating agent having a coordinating moiety linked to a central hydrophobic moiety that terminates in a hydrophilic moiety. The complex is well suited for

oral and other forms of therapeutic administration of nucleic acids in order to exact systemic and/or localized gene delivery therapy. Intestinal epithelial cells, as well as non-epithelial cells within the gastrointestinal tract and other target cells, are transformed for short or long-term therapies through oral administration, direct injection, or infusive administrations. In a preferred embodiment, a nucleic acid conjugating agent contains a bile acid linked with a polycationic peptide.

A nucleic acid conjugating agent particulate composition amenable for administration as a gene therapy composition is provided. The composition is readily adjusted to create a particle having a controlled size and net-zero, -positive, or -negative charge. The resulting composition is optionally provided in a pharmaceutically acceptable carrier for administration to achieve gene therapy, elicit an immunological response, label transfected cells, or deliver associated pharmaceutically active agents therewith.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is transmission electron microscopy (TEM) of a bile acid conjugate (BAC)/plasmid DNA (pDNA) complex;

Figures 2a-2d are relative percent particle size bar graphs of: (Fig. 2a) pDNA; (Fig. 2b) pDNA and 50 μg/ml BAC initially upon mixing and corresponding to a negatively charged complex; (Fig. 2c) pDNA and 200 μg/ml BAC initially upon mixing and corresponding to a neutrally charged complex; and (Fig. 2d) the complex depicted in Figure 2c after two hours of mixing;

Figures 3a-3c are relative percent particle size bar graphs of inventive BAC-pDNA particles having a relative ratio of BAC:pDNA of 80,699:1 in: (Fig. 3a) water; (Fig. 3b) simulated intestinal fluid; and (Fig. 3c) simulated gastric fluid;

Figure 4 is a plot of the zeta potential measured as a function of conjugating agent:DNA mol ratio where (●) indicates addition of further conjugating agent after particle formation and (▲) indicates the addition of additional nucleic acid after particle formation;

Figure 5 is an electrophoretic gel showing the relative mobility of nucleic acid, alone and in combination with at least one of conjugating agent, polyaspartic acid and DNAse I;

Figure 6 is a bar graph showing *in vitro* transfection of Hela cells with BAC-luciferase pDNA at various conjugating agent:pDNA ratios; and

Figure 7 is a plot of inventive complex single pass absorption for neutrally charged and positively charged radiolabeled BAC-pDNA as a function of time where filled symbols indicate jejunal absorption and open symbols indicate ileal absorption.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention has utility as a treatment for a variety of disease conditions or deficiencies. These conditions and deficiencies illustratively include: enzyme deficiency, erythropoietin, catalase, endotoxic shock/sepsis, adenosine deaminase for treatment of severe combined immunodeficiency, lipid-binding protein (LBP), purine nucleotide phosphorylase, galactosidase,

beta-glucuronidase, antioxidants for cancer, therapy anemia, superoxide dismutase, cancer, growth factors for use in wound healing, induction of red blood cell formation and the like, α-interferon, β-interferon, epidermal growth factor, granulocyte colony stimulating factor (G-CSF), alpha-IL1, gamma-interferon, phenylalanine ammonia lyase, transforming growth factor, arginase, erythropoietin, L-asparaginase, thrombopoietin, uricase, insulin-like growth factor-1, insulin, human growth hormone, monoclonal antibodies, tissue necrosis factor, cardiovascular disease, diabetes, tissue plasminogen activator, urokinase (native or chimeric), glucagon, α_1-antitrypsin, insulinotrophic hormone, clotting disorders, antithrombin-III, other proteases or protease inhibitors, clotting factor VIII, apolipoproteins (particularly B-48), circulating scavenger receptor, APO A1 which converts low-density lipoproteins to high-density lipoproteins, gastrointestinal and pancreatic deficiencies, obesity and feeding, pepsin (for esophageal reflux), Ob gene product, cholecystokinin (CCK), trypsin, chymotrypsin, bone diseases, elastase, carboxypeptidase, calcitonin, lactase (for lactose deficiency), PTH-like hormone, sucrase, intrinsic factor (pernicious anemia), myasthenia gravis (acetylcholine receptors), Graves' disease (thyroid-stimulating hormone receptor), organ-specific autoimmune diseases (target of antibody in parentheses), thyroiditis (thyroid, peroxidase), insulin-resistant diabetes with acanthosis nigricans or with ataxia telangiectasia (insulin receptor), allergic rhinitis, asthma (β_2-adrenergic receptors), juvenile insulin-dependent diabetes (insulin, GAD65), pernicious anemia (gastric parietal cells, vitamin B_{12} binding site of intrinsic

factor), Addison's disease (adrenal cells), idiopathic hypoparathyroidism (parathyroid cells), spontaneous infertility (sperm), premature ovarian failure (interstitial cells, corpus luteum cells), pemphigus (intercellular substance of skin and mucosa), bullous pemphigoid (basement membrane zone of skin and mucosa), primary biliary cirrhosis (mitochondria), autoimmune hemolytic anemia (erythrocytes), idiopathic thrombocytopenic purpura (platelet), idiopathic neutropenia (neutrophils), vitiligo (melanocytes), osteosclerosis and Meniere's disease (type II collagen), chronic active hepatitis (nuclei of hepatocytes), systemic autoimmune diseases (defect/organ affected in parentheses), Goodpasture's syndrome (basement membranes), rheumatoid arthritis (γ-globulin, EBV-related antigens, collagen types II and III), Sjogren's syndrome (γ-globulin, SS-A (Ro), SS-B (La), systemic lupus erythematosus (nuclei, double-stranded DNA, single-stranded DNA, Sm ribonucleoprotein, lymphocytes, erythrocytes, neurons, gamma-globulin), scleroderm (nuclei, Scl-70, SS-A (Ro), SS-B (La), centromere, polymyositis (nuclei, Jo-1, PL-7, histadyl-tRNA synthetase, threonyl-tRNA synthetase, PM-1, Mi-2), rheumatic fever (myocardium heart valves), and choroid plexus.

The invention involves methods and products for oral, parenteral, mucosal, and infusion delivery of nucleic acid for both systemic and localized therapy. Depending on the nature of the nucleic acid sequence, these contain, in non-covalently bound form, one or more substances having an affinity for nucleic acid, which are capable of increasing the efficiency of absorption of the complexes into the cells. Cells of a mammalian subject, either intestinal

epithelia after oral delivery, or cells in other organs after other forms of inventive delivery, are altered to operatively incorporate a gene which expresses a protein, which is secreted directly into the organ and/or blood stream to provide a therapeutic effect. The use of naked nucleic acid protected by complexation with adsorption and/or internalization factors avoids the complications associated with use of viral vectors to accomplish gene therapy. An inventive complex is delivered via the intestinal lumen in a variety of ways, including through timed-release capsules, such as those detailed in U.S. Patent 4,976,949, thereby obtaining a simple, noninvasive method of gene delivery. These complexes also optionally are delivered to other organs of the body in a variety of ways, including direct injection or infusion.

As used herein, a "gene" is defined to be an isolated nucleic acid molecule of greater than twenty nucleotides. A gene operative herein is recognized to be one that illustratively replaces or supplements a desired function, or achieves a desired effect such as the inhibition of tumor growth or induction of an immune response to the gene itself or a polypeptide transcribed therefrom. It is appreciated that a nucleic acid molecule according to the present invention illustratively includes plasmids, vectors, external guide sequences for RNAase, ribozymes, DNA, RNA, and miRNA. Antisense nucleic acids sequences are also administered according to the present invention. A gene is generally under the control of an appropriate promoter, which may be inducible, repressible, or constitutive. Promoters can be general promoters, yielding expression in a variety of mammalian cells, or cell specific,

or even nuclear versus cytoplasmic specific. Viral promoters such as CMV are also operative herein. These are known to those skilled in the art and can be constructed using standard molecular biology protocols.

In a preferred embodiment administration is oral and targeted to transfect intestinal epithelial cells.

As used herein, a "subject" includes humans, non-human primates, horses, goats, cows, sheep, pigs, dogs, cats, and rodents. The methods and compounds of the present invention are administered in therapeutically effective amounts.

As used herein, a "therapeutically effective amount" is defined to include an amount necessary to delay the onset of, inhibit the progress of, relieve the symptoms of, or reverse a condition being treated; induce an immune response to the delivered gene or a polypeptide encoded thereby or regulate the expression of an existing cellular product. The therapeutically effective amount is one that is less than that that produces medically unacceptable side effects. It is appreciated that a therapeutically effective amount varies with a number of factors illustratively including subject age, condition, sex and the nature of the condition being treated. It is further appreciated that determining a therapeutically effective dose is within the knowledge of one of ordinary skill in the art.

As used herein, however, the term "peptide" is intended to include mimetics and is used synonymously with polypeptide. The term "amino acid" is intended to include D-form amino acids and modified amino acids.

The compounds of the present invention are administered to a subject at dosage levels in the range of about 0.000002 mg/m^2 to about 4 mg/m^2 of conjugating agent combined with about 0.2 mg/m^2 to about 4 mg/m^2 of nucleic acid per day. For a normal human adult having a body weight of about 70 kg, a dosage in the range of about 0.005-10 mg/kg/day conjugating agent combined with about $5x10^{-6}$ -10 mg/kg/day nucleic acid is preferable. The general ratio of the amount of conjugating agent to the nucleic acid ranges from about 50:1-500,000:1 in the composition which is administered to a subject.

After oral delivery the transformed intestinal epithelial cells provide short or long term therapies for diseases associated with a deficiency in a particular protein or which are amenable to treatment or palliation by over expression of a protein including metabolic disorders, endocrine disorders, circulatory disorders, coagulation disorders, cancer, and gastrointestinal disease.

An inventive conjugating agent has the general formula:

$$A—R_1—Q—Y—Z$$

where $A—R_1$ is a cholesterol derivative; a C_8-C_{24} alkyl; C_8-C_{24} heteroatom substituted alkyl wherein the heteroatom is O, N or S; where A is a hydrophilic moiety A that illustratively includes C_0-C_4 alkyl-hydroxy, -substituted amino, -quaternary amino, -sulfonate, -phosphonate, and –carboxylate; and targeting ligand; where the targeting ligand includes amino acids, hormones, antibodies, cell adhesion molecules, folate, polypeptides, vitamins, saccharides, transferring, drugs, and neurotransmitters; where Q is

sulfur, a secondary amine, or oxygen; where Y is a linker peptide having a negative, neutral, or positive charge; and where Z is a polyionic peptide. Specific examples of inventive cholesterol derivatives illustratively include cholestanol, coprostanol, cholic acid, glycocholic acid, chenodeoxycholic acid, desoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, and taurochenodeoxycholic acid. Specific examples of C_8-C_{24} alkyls are 13-hydroxyl tridecanoic acid; 1,12 dodecane diol; and 1,12 dodecanediame.

A peptide linker sequence Y is preferably employed to separate A–R_1–Q and the polyionic peptide sequence Z that interacts with the nucleic acid by a distance sufficient to ensure that the cholesterol derivative is stearically accessible and that the polyionic peptide Z folds into its secondary and tertiary structures. Such a peptide linker sequence Y is incorporated into an inventive compound using standard techniques well known in the art. Suitable peptide linker sequences Y are chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes of the inventive compound; and (3) the lack of hydrophobic or charged residues that might react with the polyionic peptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near-neutral amino acids, such as Thr and Ala, also are operative in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., Gene 40:39-46, 1985; Murphy et al., Proc. Natl. Acad. Sci. USA 83:8258-8262, 1986; U.S. Patent Nos. 4,935,233 and 4,751,180. The

linker sequence may be from 0 to about 50 amino acids in length. A peptide linker sequence Y is not required when the polyionic peptide Z has non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

A polyionic peptide Z according to the present invention is generally a highly charged polypeptide or protein, having an isoelectric point of between about 3 to about 12. The polyionic peptide Z is generally soluble in salt-free, aqueous solution. Illustrative polyionic peptides include, but are not limited to, one or more of the following: albumin, such as from egg or animal, e.g. bovine, serum; derivatized collagen polypeptides, such as cationic collagen polypeptides; elastin; globulin polypeptide, such as myoglobin; synthetic polypeptides rich in glutamic acid, aspartic acid, lysine or arginine residues, such as polyaspartic acid; and derivatives of such proteinaceous or other materials, such as keratin. A particularly preferred polyionic peptide Z is polyaspartic acid. It is appreciated that in the formation of synthetic polyionic peptides that the inclusion of both cationic and anionic amino acid residues create a complex charge on an inventive compound under pH conditions that vary from the isoelectric point.

Proteins usefully expressed according to the administration of the present invention illustratively include proteases, pituitary hormones, protease inhibitors, growth factors, cytokines, somatomedians, chemokines, immunoglobulins, gonadotrophins, interleukins, chemotactins, interferons, and lipid-binding proteins, specific examples of which illustratively include insulin,

interferon-α2B, human growth hormone (hGH), transforming growth factor (TGF), erythropoietin (EPO), ciliary neurite transforming factor (CNTF), clotting factor VIII, insulin-like growth factor-1 (IGF-1), bovine growth hormone (BGH), granulocyte macrophage colony stimulating factor (GM-CSF), platelet derived growth factor (PDGF), interferon-α2A, clotting factor VIII, brain-derived neurite factor (BDNF), thrombopoietin (TPO), insulintropin, tissue plasminogen activator (tPA), IL-1, IL-2, urokinase, IL-1 RA, streptokinase, superoxide dismutase (SOD), adenosine deamidase, catalase, calcitonin, arginase, fibroblast growth factor (FGF) (acidic or basic), neurite growth factor (NGF), phenylalanine ammonia lyase, granulocyte colony stimulating γ-interferon factor (G-CSF), L-asparaginase, pepsin, uricase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, intrinsic factor parathyroid hormone (PTH)-like hormone, calcitonin, Ob gene product, cholecystokinin (CCK), glucagon, glucagon-like-peptide I (GLP-1), and insulinotrophic hormone.

The conjugate agent A–R_1–Q–Y–Z is preferably a bile acid conjugated with a polycationic peptide linked to the bile acid steroid backbone. The bile acid moiety acts to target the conjugate to bile acid transporters in the lumen of the intestine and assist in the cellular internalization of the complex. Short polycation peptides rich in arginine or lysine, such as a six amino acid residue or longer chain, provide multiple functions illustratively including: a) having an affinity for nucleic acid, b) act condensing agent, c) protect the nucleic acid from nuclease activity and d) assist in cellular internalization of the complex.

Conjugation of the polyionic polypeptide chain to the bile salt is made through the hydroxyl groups in the 3, 7, or 12 positions of a bile acid steroid nucleus, or on the 24-carboxyl group of the bile acids.

Composition of the polyionic peptide nucleic acid condensing moiety is altered to vary the affinity to the nucleic acid, providing changes in the rate of intracellular decondensing of the nucleic acid. These alterations are known to one skilled in the art.

Alternatively, A is a targeting ligand. A targeting ligand according to the present invention is any molecule which binds to specific types of cells. A targeting ligand may be any type of molecule having a corresponding cellular receptor. Ideally, a targeting ligand is recognized by a cellular receptor and expressed only on a specific type of cell thereby affording selectivity. Examples of targeting ligands which are operative herein include amino acids, hormones, antibodies, cell adhesion molecules, folate, polypeptides, vitamins, saccharides, transferring, drugs, and neurotransmitters. The 12-residue membrane-translocating peptide (Ala-Ala-Val-Leu-Leu-Pro-Val-Leu-Leu-Ala-Ala-Pro) (SEQ ID NO: 1) is exemplary of a target ligand form of R_1. The targeting ligand operative to either target, increase adsorption, internalization or nuclear localization of the inventive compound.

In another embodiment, the selected nucleic acid is, or encodes, an RNA molecule comprising an antisense which blocks expression of a gene, e.g., in a tumor cell. By blocking expression of this selected gene, inhibition of growth is observed for the tumor.

It is appreciated that a nucleic acid delivered according to the present invention includes a ribozyme. A ribozyme is a catalytic RNA molecule that cleaves other RNA molecules such as mRNA transcripts in a cell. Common targets include RNAs having GUC or GUA subsequences. For example, hairpin ribozymes typically cleave one of two target sequences. GUC hairpin ribozymes cleave an RNA target sequence consisting of NNNBCN*G UCNNNNNNNN (SEQ ID NO: 2) where N*G is the cleavage site, B is any of G, U or C, and where N is any of G, U, C, or A. GUA ribozymes typically cleave an RNA target sequence consisting of NNNNN*GUANNNNNNNN (SEQ ID NO: 3) where N*G is the cleavage site and where N is any of G, U, C, or A. See, De Young et al. (1995) Biochemistry 34: 15785-15791. Ribozymes delivered optionally have non-standard ribonucleotide bases, or deoxyribonucleotide bases, which can stabilize the ribozyme and make the ribozyme resistant to RNase enzymes. Alternatively, the ribozyme is modified to a phosphothio analog, thereby rendering the ribozyme resistant to endonuclease activity.

According to the present invention, a systemic effect is achieved by transducing cells with genes that express proteins that are secreted into the circulatory system and therefore provide a systemic effect. As is known to one skilled in the art, deleting from a gene pro-protein sequences or transporter sequences tends to restrict a protein to an intracellular effect. Additionally, according to the present invention gene expression and therefore therapeutic effect is regulated through administration. In an embodiment where intestinal

epithelial cells are transduced, these cells are sloughed from the lumen every several days thereby affording only transient gene expression. Conversely, the transduction of cells with less turnover than intestinal epithelial cells affords longer gene expression periods.

The compounds of the present invention can be administered to a patient either alone or a part of a pharmaceutical composition. The compositions can be administered to patients either orally, rectally, parenterally (intravenously, intramuscularly, or subcutaneously), intracisternally, intravaginally, intreperitoneally, intravesically, locally (powders, ointments, or drops), or as a buccal or nasal spray.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents,

for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol, and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose, and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates, and sodium carbonate, (e) solution retarders, as for example, paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol, and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, or mixtures thereof. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like.

Solid dosage forms such as tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedding compositions which can be used are polymeric substances and waxes. The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays, and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and preservatives, buffers, or propellants as may be required. Ophthalmic formulations, eye ointments, powders, and solution are also contemplated as being within the scope of this invention.

An inventive compound is also delivered in conjunction with an active therapeutic compound, a pharmaceutically acceptable salt, ester, amide or prodrug. The therapeutic compound illustratively being active as antibiotic, a

gamma or beta radiation emitting species, an anti-inflammatory, an antitumoral, an antiviral, an antibody, a hormone, an enzyme, and antigenic peptide or protein.

The term "pharmaceutically acceptable salts, esters, amides, and prodrugs" as used herein refers to those carboxylate salts, amino acid addition salts, esters, amides, and prodrugs of the compounds of the present invention which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of patients without undue toxicity, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use, as well as the zwitterionic forms, where possible, of the compounds of the invention. The term "salts" refers to the relatively non-toxic, inorganic and organic acid addition salts of compounds of the present invention. These salts can be prepared in situ during the final isolation and purification of the compounds or by separately reacting the purified compound in its free base form with a suitable organic or inorganic acid and isolating the salt thus formed. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, nitrate, acetate, oxalate, valerate, oleate, palmitate, stearate, laurate, borate, benzoate, lactate, phosphate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate mesylate, glucoheptonate, lactobionate and laurylsulphonate salts, and the like. These may include cations based on the alkali and alkaline earth metals, such as sodium, lithium, potassium, calcium, magnesium, and the like, as well as non-toxic ammonium, quaternary ammonium and amine cations including, but

not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. (See, for example, S.M. Barge et al., "Pharmaceutical Salts," *J. Pharm. Sci.*, 1977, 66:1-19 which is incorporated herein by reference.)

The examples presented below are intended to illustrate particular embodiments of the invention and are not intended to limit the scope of the specification, including the claims.

EXAMPLES

Example 1

Method for synthesis of bile acid conjugates (BAC)

BAC is synthesized by solid phase chemistry on a peptide synthesizer. A six L-arginine peptide is first synthesized on the resin bed using standard 9-fluorenylmethoxycarbonyl (FMOC) chemistry. To attach the bile acid salt, an excess of chendoxycholic acid is added to the resin and allowed to react with the immobilized peptide. After conjugation, BAC is cleaved from the resin and purified to greater than 95% purity by HPLC.

Example 2

Construction of Expression Plasmids

An expression plasmid using an optimized Cytomegalovirus (CMV) early promoter-enhancer to drive transgene expression (pCF1) is utilized. For cell localization and transfection expression experiments the firefly luciferase cDNA is used as a reporter transgene (pCF1-luc). Plasmid backbones which are chosen to contain the origin of replication and an antibiotic resistance gene

or other selectable marker allowing the growth and maintenance of the plasmid in its bacterial host. Other attributes to facilitate the production of plasmid DNA are: 1) the ability of the plasmid to produce high copy number in the host bacteria; 2) a eukaryotic transcriptional promoter to initiate the synthesis of mRNA when it enters the target cells; 3) the DNA coding sequence encoding the protein of interest with a translational start codon (ATG) in the sequence for initiation of protein synthesis and a stop codon to terminate translation; and 4) an optional transcription termination sequence.

Example 3

Preparation of BAC/pDNA Complexes

Typically an equal volume of BAC solution at 3 mg/ml (2.25 mM) is slowly added with mixing to a pDNA solution at a concentration of 250μg/ml to form a neutrally charged complex. Mass spec analysis has provided evidence that the major ionic species in the BAC has a charge of +3 per BAC molecule (MW = 1329) under neutral conditions (data not shown). Assuming a charge of –2 per base pair of the 4550 bp pDNA (MW = 3,003,000) (total DNA charge = -9100), a net neutral charge would theoretically require a ratio of about 3033:1 (BAC:pDNA). Physical measurements of the BAC/pDNA complex appear require about 10-fold more BAC to neutralize the pDNA charge (see Table 1). The presence of buffer salts bound to the BAC during synthesis and purification may partially explain this observation.

Table 1

BAC amount µg/ml	BAC:pDNA molar ratio	Particle Charge determined by Zeta Potential
50	8070:1	Negative
200	32,280:1	Neutral
2000	322,799:1	Positive

Example 4

BAC condenses plasmid DNA.

The ability of BAC to condense DNA is determined using transmission electron microscopy (TEM). TEM of a BAC/pDNA particle complex is shown in Figure 1. The mole ratio of BAC:pDNA is 1581:1, which retained a net negative charge on the particle. The samples after mixing are diluted in water, and a drop applied to the carbon-coated formvar-film grid. The samples are dried and stained with uranyl acetate before viewing. Length bars in Figure 1 are 100 nM long. Even at this low molar ratio of BAC:pDNA clear rod shaped particles (toroids) are formed.

Example 5

BAC condensed plasmid DNA forms discrete particles.

NICOMP particle size analysis is shown in Figure 2. 1 ml of 14 µg/ml DNA plasmid is mixed with 1 ml of BAC at the concentrations shown in Table 1. Also shown in Table 1 are the calculated molar ratios and particle charges.

Particle sizes are measured at a specific time after mixing of the pDNA with the BAC.

Figures 2(a)-(d) show some representative results of these experiments. Mixtures of pDNA and BAC start forming particles immediately. However, with increasing time these particles appear to aggregate, so that at later times particles larger than 1 μm are observed. Figure 2(a) is particle sizing for the plasma DNA of Example 2. Figure 2(b) shows the particle size distribution of 1 ml of 14 microgram per ml DNA plasmid mixed with 1 ml of 50 microgram per ml BAC to yield a negatively charged complex which is sized immediately upon mixing. Figure 2(c) shows a particle size distribution as per Figure 2(b) except 200 microgram per ml BAC is introduced instead of 50 microgram per ml BAC to yield a neutrally charged complex. Figure 2(d) shows the particle size distribution for the experiment according to Figure 2(c) two hours after mixing.

Example 6

BAC condensed plasmid DNA particle size in simulated gastric or intestinal fluid.

The behavior of the pDNA/BAC complex in buffers with different pHs to simulate gastric fluid (SGF) or intestinal fluid (SIF) are evaluated. pDNA and BAC (BAC:pDNA: = 80,699:1, slightly positive charge) are mixed first in water and then mixed with the same volume of simulated gastric fluid (SGF, 0.1 M HCl, pH 1.2) or simulated intestinal fluid (SIF, 0.05 M phosphate buffer, pH 6.8). Measurements are made after two hours incubation at 37°C. After 2 hours, most of the DNA complexes in water (Figure 3a) and SIF (Figure 3b) are observed to be greater than 1 um in size. However, over 30% of DNA complex incubated in SGF (Figure 3c) is observed to be under 1 μm size.

These results indicate that pH has some effect on the physical behavior of BAC/pDNA complexes.

Example 7

Measurement of the zeta potential (net charge) of the particle complexes are made after mixing BAC/pDNA in water. 800 µl of 11.07 microgram/ml DNA plasmid is mixed with 800 µl of 50 µg/ml BAC (DNA:BAC = 1:10,205 -negatively charged). 10 µl of 500 µg/ml or 1 mg/ml BAC is added and zeta potential is remeasured in the first experiment (•). 200 microliters of 10 micrograms/ml pDNA is prepared and 2 microliters of 3 mg/ml BAC is added before zeta potential remeasurement in the second experiment (π). Additional aliquots of BAC are added and the zeta potential remeasured (Figure 4).

Example 8

DNase protection assays - The ability for BAC complex formation to protect DNA from endonuclease activity is examined. We have utilized the ability of polyanions such as polyaspartic acid (PAA) to competitively dissociate the polycations from DNA. Katayose S, Kataoka K. *Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer*. Bioconjug Chem 1997;8:702-7.

1) The mixture of luciferase BAC/pDNA is stored at room temperature for 2.5 hours (BAC/pDNA: = 9038:1).
2) 10 µl of 10 mg/ml PAA or milli-Q water is added to each sample (5 µl of DNA complex mixture) and stored at room temperature for 2 hours.

3) The molar ratio is (DNA:BAC:PAA= 1:9038:13,572), the charge ratio (DNA:BAC:PAA=1:3:388), assuming BAC has 3 positive charges and PAA has 260 negative charges.

4) 5 ul of 100 kunit/ml DNaseI or milli-Q water is added to each sample and those samples are incubated at 37°C for 15 min and heated at 70°C for 15 min.

5) 2 ul of 500 mM EDTA and 1 ul of 5% SDS is added to each sample and heated at 55°C for 3 hours to dissociate the BAC from the pDNA. Samples are then electrophoresed in an agarose gel and stained with ethidium bromide (Figure 5).

Extremely harsh conditions are required to dissociate BAC from the BAC/pDNA complex (1% SDS at 55°C for 2 hours). One hour is not enough to reach a plateau dissociation level.

DNaseI together with BAC in the mixture altered the effect the dissociation by SDS (Figure 5, Lane 2). The DNA sample which is incubated at 37°C for 15 min with DNaseI showed almost complete fragmentation (Figure 5, Lane 3). The BAC/pDNA sample incubated in the presence of PAA and DNAseI showed less intact DNA banding (Figure 5, Lane 1) than BAC/pDNA samples with or without treatment with PAA (Figure 5, Lanes 4 and 5 respectively).

Example 9

***In Vitro* Transfection Assays:** To examine the capability of the BAC/pDNA complexes to transfect cells *in vitro*, the ability of BAC to

transfect Hela tissue cultures using the firefly luciferase gene is examined. In transient transfection assays (24 h), BAC enhanced the transfection efficiency in combination with liposomes (data not shown). In accordance with the present invention, BAC/pDNA is able to transfect Hela cells alone as a single agent (Figure 6). The charge ratio is approximately neutral. The approximate molar ratios are indicated in parenthesis.

Example 10

***In Situ* Intestinal Absorption:** In order to examine the capability of the BAC/pDNA complex to bind and/or be absorbed onto the intestinal lumen cells the absorption of nicked-radiolabeled pDNA in a single pass perfusion experiment is tested. Using isolated ileal or jejunal segments radio-labeled BAC/pDNA mixtures mixture is mixed with HEPES buffer, pH 7.4, and perfused at jejunal (filled symbols) and ileal (open symbols) sites. After 30 min of steady state, sample fractions are collected and the absorbed fraction of BAC/pDNA calculated. BAC/pDNA mixtures used are pDNA:BAC 1:27,000 neutrally charged and 1:54,000 positively charged. Uncomplexed pDNA is used as a control.

Between 20-40% of the "neutrally charged" BAC/pDNA complex is absorbed in a single pass of the intestine as shown in Figure 7. As a result, it is appreciated that this complex should be completely absorbed after oral dosing.

Example 11

***In Situ* Intestinal Absorption for Translocating Peptide BAC:** The 12-residue membrane translocating peptide (SEQ ID NO: 1) is coupled to the

BAC by way of a condensation reaction involving the three alpha hydroxyl group. The complexes as detailed in Example 10 are recreated with the membrane translocating BAC yielding comparable results to those depicted in Figure 7.

Example 12

***In Situ* Intestinal Absorption for Translocating Peptide BAC:** The 12-residue membrane translocating peptide (SEQ ID NO: 1) is coupled to the BAC by way of a condensation reaction involving the three alpha hydroxyl group. The complexes as detailed in Example 10 are recreated with the membrane translocating BAC yielding comparable results to those depicted in Figure 7.

Patent applications and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These applications and publications are incorporated herein by reference to the same extent as if each individual application or publication was specifically and individually incorporated herein by reference.

The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the invention.

What is claimed is:

CLAIMS

1. A method for treating a disease condition or deficiency through gene delivery to target cells of a subject comprising the step of administering a conjugating agent-nucleic acid complex where the conjugating agent comprises R_1–Z, where R_1 is a cholesterol derivative; a C_8-C_{24} alkyl; C_8-C_{24} heteroatom substituted alkyl wherein the heteroatom is O, N, or S; or membrane importer and transporter proteins and Z is a polyionic peptide.

2. The method of claim 1, wherein said administration is oral.

3. The method of claim 1, wherein nucleic acid of said complex is expressed as a protein in said target cells.

4. The method of claim 3 wherein said protein is secreted from said target cells.

5. The method of claim 3 wherein said protein is of a class selected from the group consisting of: proteases, pituitary hormones, protease inhibitors, growth factors, cytokines, somatomedians, chemokines, immunoglobulins, gonadotrophins, interleukins, chemotactins, interferons, and lipid-binding proteins.

6. The method of claim 1 wherein said nucleic acid of said complex is selected from the group consisting of: DNA, RNA, mRNA, miRNA, ribozyme, RNAse, and antisense sequences.

7. The method of claim 1 wherein said complex is administered as part of a pharmaceutical composition.

8. The method of claim 7 wherein said pharmaceutical composition comprises an active therapeutic compound.

9. The method of claim 8 wherein said therapeutic agent is selected from the group consisting of: an antibiotic, a gamma or beta radiation emitting species, an anti-inflammatory, an antitumoral, an antiviral, an antibody, a hormone, an enzyme, antigenic peptide and antigenic protein.

10. The method of claim 1 wherein R_1 is a cholesterol derivative.

11. The method of claim 10 wherein said cholesterol derivative is attached to a hydrophilic moiety A.

12. The method of claim 1, wherein said target cells are gastrointestinal cells.

13. A gene delivery composition comprising a conjugating agent-nucleic acid complex having the formula:

$$A—R_1—Q—Y—Z$$

where $A—R_1$ is a cholesterol derivative; a C_8-C_{24} alkyl; C_8-C_{24} heteroatom substituted alkyl wherein the heteroatom is O, N or S; where A is a hydrophilic moiety A that illustratively includes C_0-C_4 alkyl-hydroxy, -substituted amino, -quaternary amino, -sulfonate, -phosphonate, and –carboxylate and a target ligand; where Q is sulfur, nitrogen, or oxygen; where Y is a linker peptide having a negative, neutral, or positive charge; and where Z is a polyionic peptide.

14. The composition of claim 13 wherein said cholesterol derivative is selected from the group consisting of: cholestanol, coprostanol, cholic acid, glycocholic acid, chenodeoxycholic acid, desoxycholic acid, glycochenodeoxycholic acid, taurocholic acid, and taurochenodeoxycholic acid.

15. The composition of claim 13 wherein said cholesterol derivative is a cholic acid or a deoxycholic acid.

16. The composition of claim 13 wherein said A derivative is hydroxyl.

17. The composition of claim 13 wherein said Q derivative is oxygen.

18. The composition of claim 13 wherein Y and Z together yield a net neutral charge.

19. The composition of claim 13 wherein Z is polycationic.

20. The composition of claim 19 wherein Z contains at least six residues, each residue independently selected from the group consisting of: arginine, lysine, an amino acid having a pendent amine group, and a beta amino acid having a pendent amine group.

21. The composition of claim 13 wherein Z is polyanionic.

22. The composition of claim 21 wherein Z contains at least six residues, each residue independently selected from the group consisting of: aspartic acid, glutamic acid, an amino acid having a pendent carbonyl group, and a beta amino acid having a pendent carbonyl group.

23. Use of a bile acid salt as a conjugating agent to administer nucleic acid to a subject.

24. The use of claim 23 wherein administration is oral.

25. A commercial package comprising a composition of Formula I according to claim 1 as an active ingredient together with instructions for the use thereof as a gene delivery agent to a subject.

ABSTRACT OF THE DISCLOSURE

A method is provided for the packaging of a nucleic acid with a chelating agent having a coordinating moiety linked to a central hydrophobic moiety that terminates in a hydrophilic moiety. The complex is well suited for oral and other forms of therapeutic administration of nucleic acids in order to exact systemic and/or localized gene delivery therapy. Intestinal epithelial cells, as well as non-epithelial cells within the gastrointestinal tract and other target cells, are transformed for short or long-term therapies through oral administration, direct injection, or infusive administrations. A nucleic acid conjugating agent particulate composition amenable for administration as a gene therapy composition is provided. The composition is readily adjusted to create a particle having a controlled size and net-zero, -positive, or -negative charge.

Docket No.
TSR-10002/38

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, or plant breeder's rights certificate(s), or 365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
_____	_____	_____	☐
(Number)	(Country)	(Day/Month/Year Filed)	
_____	_____	_____	☐
(Number)	(Country)	(Day/Month/Year Filed)	
_____	_____	_____	☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/425,379	November 12, 2002
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Ernest L. Gifford, 20,644
Allen M. Krass, 18,277
Irvin L. Groh, 17,505
Douglas W. Sprinkle, 27,394
Thomas E. Anderson, 31,318
Ronald W. Citkowski, 31,005
Judith M. Riley, 31, 561
Douglas J. McEvoy, 34,385
John G. Posa, 37,424
Avery N. Goldstein, 39,204
Douglas L. Wathen, 41,369
Mark D. Schneider, 43,906
Beverly M. Bunting, 36,072
Lionel D. Anderson, 50,571
Angela M. Davison, 54,058
Martin S. Bancroft, 43,316
Julie K. Staple, 50,434

Send Correspondence to: **Avery N. Goldstein**
Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C.
280 N. Old Woodward Avenue, Suite 400
Birmingham, MI 48009-5394

Direct Telephone Calls to: *(name and telephone number)*
Avery Goldstein at (248) 647-6000

Full name of sole or first inventor
John Hilfinger

Sole or first inventor's signature — Date: 11/11/03

Residence
Ann Arbor, Michigan

Citizenship
US

Post Office Address
2636 Essex

Ann Arbor, MI 48104

Full name of second inventor, if any
Blake Roessler

Second inventor's signature — Date

Residence
Ann Arbor, Michigan

Citizenship
US

Post Office Address
3175 Dolph Drive

Ann Arbor, MI 48103

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Ernest I. Gifford, 20,644
Allen M. Krass, 18,277
Irvin L. Groh, 17,505
Douglas W. Sprinkle, 27,394
Thomas E. Anderson, 31,318
Ronald W. Citkowski, 31,005
Judith M. Riley, 31, 561
Douglas J. McEvoy, 34,385
John G. Posa, 37,424
Avery N. Goldstein, 39,204
Douglas L. Wathen, 41,369
Mark D. Schneider, 43,906
Beverly M. Bunting, 36,072
Lionel D. Anderson, 50,571
Angela M. Davison, 54,058
Martin S. Bancroft, 43,316
Julie K. Staple, 50,434

Send Correspondence to: Avery N. Goldstein
Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C.
280 N. Old Woodward Avenue, Suite 400
Birmingham, MI 48009-5394

Direct Telephone Calls to: *(name and telephone number)*
Avery Goldstein at (248) 647-6000

Full name of sole or first inventor John Hilfinger
Sole or first inventor's signature — Date
Residence Ann Arbor, Michigan
Citizenship US
Post Office Address 2636 Essex
Ann Arbor, MI 48104

Full name of second inventor, if any Blake Hannaford
Second inventor's signature — Date 11/11/03
Residence Ann Arbor, Michigan
Citizenship US
Post Office Address 3175 Dolph Drive
Ann Arbor, MI 48103

Full name of third inventor, if any
Phillip Kish

Third inventor's signature — Date
11/16/2003

Residence
Ann Arobr, Michigan

Citizenship
US

Post Office Address
4785 Lake Court

Ann Arbor, MI 48103

Full name of fourth inventor, if any

Fourth inventor's signature — Date

Residence

Citizenship

Post Office Address

Full name of fifth inventor, if any

Fifth inventor's signature — Date

Residence

Citizenship

Post Office Address

Full name of sixth inventor, if any

Sixth inventor's signature — Date

Residence

Citizenship

Post Office Address

FORD, KRASS, GROH, SPRINKLE, ANDERSON & CITKOWSKI PC

56876

OR: 230 DIRECTOR, U.S. PATENT CHECK NO:

REF. NO.	YOUR INVOICE NUMBER	INVOICE DATE	INVOICE AMOUNT	AMOUNT PAID	DISCOUNT TAKEN
39185	TSR-10002/38 JK	11/11/03	473.00	473.00	.00

Express Mail Label No. EL983172102US

heck Total 473.00

56876

GIFFORD, KRASS, GROH, SPRINKLE,
ANDERSON & CITKOWSKI PC
280 N. OLD WOODWARD, SUITE 400
BIRMINGHAM, MI 48009
248-647-6000

COMERICA BANK
DETROIT, MICHIGAN 48275
9-9-720

CHECK NO.	CHECK DATE	VENDOR NO.
056876	11/11/03	230

CHECK AMOUNT

FOUR HUNDRED SEVENTY-THREE AND 00/100 DOLLARS***** $**********473.00

Details on back. Security Features Included.

HE
ER DIRECTOR, U.S. PATENT
AND TRADEMARK OFFICE

MP

⑈056876⑈ ⑆072000096⑆ 1840129546⑈

FORD, KRASS, GROH, SPRINKLE, ANDERSON & CITKOWSKI PC

56876

230 DIRECTOR, U.S. PATENT

39185 TSR-10002/38 JK 11/11/03 473.00 473.00 .00

heck Total 473.00

PATENT MS: PAT. APPL.

The received stamp of the U.S. Patent and Trademark Office imprinted hereon acknowledges the filing of:

☒ Transmittal Letter ☒ New Patent Application (claims prov.)

27 Page(s) Specifications 5 Page(s) Claims

1 Page(s) Abstract 7 Sheets of Drawings

☐ Small Entity Declaration ☒ Declaration & Power of Atty

☐ IDS, PTO 1449 & Patents ☐ Assignment & Recordation Cover Sheet

☐ Amendment/Response ☒ Check #473.00

Other: Application Data Sheet

Inventor: John Hilfinger et al.

Serial/Reg. No. _____ Atty Docket No. TSR-10002/38

Date: Nov. 12, 2003 Date Due: Nov. 12, 2003

☒ Cert. Of ~~Mailing~~/Express Mail Label No. EL983172102US

ANG/JK

EXPRESS MAIL
UNITED STATES POSTAL SERVICE®
www.usps.com

EXPRESS MAIL
UNITED STATES POSTAL SERVICE®
CORPORATE ACCOUNT
POSTAGE AND FEES PAID
Label 108 May 2000

EL 983172102 US

Mailing Label
Label 11-F June 2002

EXPRESS MAIL
UNITED STATES POSTAL SERVICE®

Post Office To Addressee

ORIGIN (POSTAL USE ONLY)			
PO ZIP Code	Day of Delivery ☐ Next ☐ Second	Flat Rate Envelope ☐	
Date In Mo. Day Year	☐ 12 Noon ☐ 3 PM	Postage $	
Time In ☐ AM ☐ PM	Military ☐ 2nd Day ☐ 3rd Day	Return Receipt Fee	
Weight lbs. ozs.	Int'l Alpha Country Code	COD Fee	Insurance Fee
No Delivery ☐ Weekend ☐ Holiday	Acceptance Clerk Initials	Total Postage & Fees $	

DELIVERY (POSTAL USE ONLY)		
Delivery Attempt Mo. Day	Time ☐ AM ☐ PM	Employee Signature
Delivery Attempt Mo. Day	Time ☐ AM ☐ PM	Employee Signature
Delivery Date Mo. Day	Time ☐ AM ☐ PM	Employee Signature

☐ WAIVER OF SIGNATURE *(Domestic Only)* Additional merchandise insurance is void if waiver of signature is requested. I wish delivery to be made without obtaining signature of addressee or addressee's agent (if delivery employee judges that article can be left in secure location) and I authorize that delivery employee's signature constitutes valid proof of delivery.

NO DELIVERY ☐ Weekend ☐ Holiday

Customer Signature

CUSTOMER USE ONLY

METHOD OF PAYMENT:
Express Mail Corporate Acct. No. X480320

Federal Agency Acct. No. or Postal Service Acct. No.

FROM: (PLEASE PRINT) PHONE 248 647 6000

GIFFORD KRASS GROH & ASSOC PC
280 N OLD WOODWARD AVE STE 400
BIRMINGHAM MI 48009-5394

ANG/JK TSR-10002/38

TO: (PLEASE PRINT) PHONE ( )

MAIL STOP PATENT APPLICATION
COMMISSIONER FOR PATENTS
PO BOX 1450
ALEXANDRIA VA 22313-1450

PRESS HARD.
You are making 3 copies.

FOR PICKUP OR TRACKING CALL 1-800-222-1811 www.usps.com

EMS

EP-13F February 2002

PATENT MS: PAT. APPL.

The receive[illegible]mp of the U.S. Patent and Trademark Office [illegible]inted hereon acknowledges the filing of:

☒ Transmittal Letter ☒ New Patent Application (claims prop

27 Page(s) Specifications 5 Page(s) Claims

1 Page(s) Abstract 7 Sheets of Drawings

☐ Small Entity Declaration ☒ Declaration & Power of Atty

☐ IDS, PTO 1449 & Patents ☐ Assignment & Recordation Cover Sheet

☐ Amendment/Response ☒ Check #473 22141 U.S. PTO 10/706738

Other: Application Data Sheet

111203

Inventor: John Hilfinger et al.

Serial/Reg. No. _____ Atty Docket No. TSR-1006[illegible]

Date: Nov. 12, 2003 Date Due: Nov. 12, 2003

☒ Cert. Of ~~Mailing~~/Express Mail Label No. EL983172102US

ANG/JK

The PTO did not receive the following listed item(s)

Date of Claims

Attorney Docket No. TSR-10002/38

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: John Hilfinger et al.

Serial No.: 10/706,738

Filing Date: November 12, 2003

For: METHODS AND COMPOSITIONS OF GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

DECLARATION OF JANICE R. KUEHN

Janice R. Kuehn declares as follows:

1. I am a legal assistant for the patent law firm of Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C. I have been employed by this firm since November 1996.

2. In my present position I am secretary to patent attorneys Avery N. Goldstein and Angela M. Davison. In that capacity I prepare patent application submission documents to be filed with the U.S. Patent and Trademark Office.

3. In my capacity, when preparing a United States utility patent application, I print final versions of the transmittal pages, application data sheets, specification, claims and abstract. I also assemble the declarations and drawings which may or may not be in digital form. It is a standard procedure that the specification, claims and abstract are all contained within a single document in Word format (Microsoft Corporation). The transmittal pages and application data sheet are prepared using LegalStar IP Forms and EPAVE software from the USPTO, respectively. The computer-printed documents are always the documents submitted to the Patent Office, with copies being made for our file and the client. Thus, if a copying error occurred, it

would appear in our file, not at the Patent Office. In this instant case, the file copy and client copy are complete.

4. In the process of preparing the transmittal papers, necessary to fill out the fee calculation and transmittal fields I prepare a tally of the total number of claims, total number of independent claims, and the numbering of the independent claims. On this same piece of paper I also run a tally of total numbers of pages corresponding to transmittal papers, declaration, application data sheet, specification, claims, abstract and drawings.

5. In preparing to actually mail an application to the United States Patent and Trademark Office I use this tally sheet to finally count the number of pages corresponding to each portion of the submission and further to fill out the return receipt postcard that accompanies the submission. The tally sheet used in this particular patent application submission is attached at Appendix 1 to this declaration. The attached tally sheet includes an error of 28 pages of specification, 5 pages of claims, and one page of abstract. As this tally sheet was used to fill out the transmittal pages, page 1, section 2 noting the specification having 34 pages, the error as to the specification having 28 pages instead of the actual number of 27 appears in the line stating that the specification has 34 pages. Additionally, the total pages in the submission noted as being 52 in the upper left-hand corner of the transmittal pages indicating 52 pages is likewise in error based on a miscount at the tally sheet stage of the submission preparation.

6. The final document of the submission package I complete is the return receipt postcard for which I perform a separate count of specification pages, claim pages, pages of abstract, and drawing pages. While it is certainly possible as happened in this case that a one page discrepancy in a specification may not be caught in this final count, I cannot imagine a

scenario by which an entire section, namely five pages of claims constituting the full set of claims, would be omitted from a utility patent application submission.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject patent.

Janice R. Kuehn

Janice R. Kuehn

Date: April 13, 2004

ANG/gs
GS-W:\Word Processing\ang\TSR10002-KuehnDec.doc

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
Washington, DC 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
60/425,379	11/12/2002		80	TSR-10081/38	7		

CONFIRMATION NO. 4964

25006
GIFFORD, KRASS, GROH, SPRINKLE
ANDERSON & CITKOWSKI, PC
280 N OLD WOODARD AVE
SUITE 400
BIRMINGHAM, MI 48009

FILING RECEIPT

OC000000009261588

Date Mailed: 12/18/2002

Receipt is acknowledged of this provisional Patent Application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

Phillip Kish, Ann Arbor, MI;

If Required, Foreign Filing License Granted: 12/18/2002

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

**** SMALL ENTITY ****

Title

Methods and compositions gene delivery agents for sytemic and local therapy

---

LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

TSR-10081/38
21111gs/jk

METHODS AND COMPOSITIONS GENE DELIVERY AGENTS FOR SYSTEMIC AND LOCAL THERAPY

Field of the Invention

The present invention relates to gene therapy and more particularly to drug delivery molecules for delivering various nucleic acids compositions to the lumen of the intestine or to localized tissues.

Background of the Invention

Gene therapy has garnered considerable attention as a method to treat various human diseases by the enhancement of protein production. These include gene replacement or gene augmentation.

This invention allows the performance of *in vivo* gene therapy by oral and other methods.

This invention provides for a method to perform systemic therapy for various mammalian conditions. Tables 2-4 in the Appendix describe some of the possible proteins, classes of proteins and potential disease conditions amenable to gene therapy.

Methodology review of other gene therapy delivery strategies, which utilize mechanical direct injection or particle bombardment, recombinant viruses and liposomes that are dependent on receptor-mediated endocytosis.

Oral therapy is easy to administer, generally the least expensive, and has good patient compliance for dosing.

Difficulties in this method:

Epithelial cells have a 2-4 day turnover rate, meaning short duration of expression of transformed cells.

Mucus layer may further reduce the amount of the cellular exposure and reduce transformation efficiency.

The intestine routinely degrades large amounts of foreign DNA ingested in our food. The presence of DNAses in the intestinal tract can provide a significant barrier to entry of intact DNA into intestinal tract cells.

Brief Description of the Drawings

Figure 1. Transmission electron microscopy (TEM) of BAC/pDNA complex.

Figure 2. Measurement of BAC / plasmid DNA particle formation.

Figure 3. Measurement of BAC condensed plasmid DNA particle size in simulated gastric or intestinal fluid.

Figure 4. BAC/pDNA Particle Zeta Potential Measurements.

Figure 5. Endonuclease Protection Assay.

Figure 6. *In vitro* transfection of Hela cells with BAC/luciferase pDNA.

Figure 7. *In situ* perfusion of radiolabeled BAC/pDNA in intestinal segments.

Detailed Description of the Preferred Embodiments

The invention involves methods and products for oral and parenteral gene delivery for both systemic and localized therapy. These contain, in non-covalently bound form, one or more substances having an affinity for nucleic acid, which are capable of increasing the efficiency of absorption of the complexes into the cells. Cells of a mammalian subject (either intestinal

epithelia after oral delivery, or cells in other organs after parenteral delivery) are altered to operatively incorporate a gene, which expresses a protein, which is secreted directly into the organ and/or blood stream to provide a therapeutic effect. The use of naked nucleic acid protected by complexation with adsorption/internalization factors avoids the complications associated with use of viral vectors to accomplish gene therapy. These complexes can be delivered via the intestinal lumen in a variety of ways, including through timed-release capsules, thereby obtaining a simple, noninvasive method of gene delivery for gene therapy. These complexes can also be delivered to other organs of the body in a variety of ways, including direct injection or infusion. After oral delivery the transformed intestinal epithelial cells provide short or long term therapies for diseases associated with a deficiency in a particular protein or which are amenable to treatment or palliation by over expression of a protein including metabolic disorders, endocrine disorders, circulatory disorders, coagulation disorders, cancer, and gastrointestinal disease.

The targeting-internalizing conjugate is preferably a bile acid conjugated with a poly cationic peptide linked to the bile acid steroid backbone. The bile acid moiety acts to target the conjugate to bile acid transporters in the lumen of the intestine and assist in the cellular internalization of the complex. Short polycation peptides rich in arginine (e.g. 6-arginine amino acids) provide multiple functions; a) having an affinity for nucleic acid, b) act condensing agent, c) protect the nucleic acid from nuclease activity and d) assist in cellular internalization of the complex.

Conjugation of the cationic polypeptide chain to the bile salt can be made through the hydroxyl groups in the 3, 7, or 12 positions of the bile acid steroid nucleus, or on the 24-carboxyl group of the bile acids.

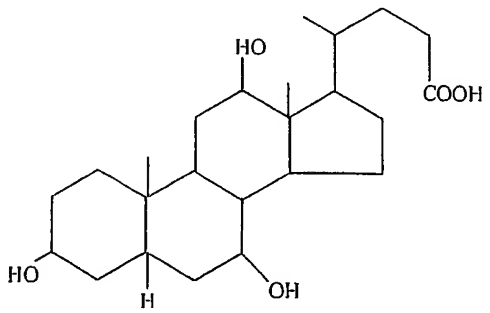
Composition of the polypeptide nucleic acid condensing moiety can be altered to vary the affinity to the nucleic acid, providing changes in the rate of intracellular decondensing of the nucleic acid. The number of amino acids in the polypeptide can be varied and be composed primarily of arginine with neutral and acidic amino acids to act as spacers and linkers to the bile acids.

One aspect of the invention relates to cationic derivatives of the bile salt acids such as cholic acid with peptides containing multiple arginine amino acids as in Formula I.

$$R_1\text{–O—(X)—Z} \qquad \text{Formula I}$$

wherein:

R_1 is cholic acid (5β-CHOLANIC ACID-3α, 7α, 12α - TRIOL) or one of its analogues;



Structure of cholic acid

X is a linker peptide with either a negative, neutral or positive charge

Z is a cationic peptide

In related aspects, the invention provides polyanion-bile acid complexes comprising the bile acid – amino acid conjugates of Formula I, methods of

their preparation and their use in delivering biologically active substances to cells.

There are several advantages of the present invention:

One advantage is that expressed protein can be secreted into the circulatory system to provide a systemic effect.

Another advantage of the present invention is that the gene expression can be regulated by the administration of the invention. Because the epithelia of the intestine are sloughed into the lumen every few days, the gene expression remains transient.

Another advantage is that the potential side effects, which may be severe, are avoided, or minimized by the rapid turnover of intestinal epithelial cells. Additionally, the use of naked DNA with a chemical conjugate avoids the antigenic reactions associated with gene therapy with viral vectors or electroporation of DNA into cell.

Another advantage of this invention is that the method completely avoids invasive procedures. The nucleic acids are orally administered in the form of a capsule, pill or other oral preparation.

In another embodiment, the selected nucleic acid is, or encodes, an RNA molecule comprising an *antisense* which blocks expression of a gene, e.g., in a tumor cell. By blocking expression of this selected gene, inhibition of growth is observed for the tumor.

In another embodiment, this oral delivery system can deliver ribozymes. A ribozyme is a catalytic RNA molecule that cleaves other RNA

molecules (e.g., mRNA transcripts in a cell) having particular nucleic acid sequences. Common targets include RNAs comprising GUC or GUA subsequences. For example, hairpin ribozymes typically cleave one of two target sequences. GUC hairpin ribozymes cleave an RNA target sequence consisting of NNNBCN*GUCNNNNNNNN (SEQ ID NO: 1) (where N*G is the cleavage site, B is any of G, U or C, and where N is any of G, U, C, or A). GUA ribozymes typically cleave an RNA target sequence consisting of NNNNN*GUANNNNNNNN (SEQ ID NO: 2) (where N*G is the cleavage site and where N is any of G, U, C, or A). See, De Young et al. (1995) Biochemistry 34: 15785-15791. Ribozymes optionally comprise non-standard ribonucleotide bases, or deoxyribonucleotide bases, which can stabilize the ribozyme and make it resistant to RNase enzymes. Alternatively, the ribozyme can be modified to a phosphothio analog for use in this *delivery* system. This modification also renders the ribozyme resistant to endonuclease activity.

In another embodiment of the present invention, incorporation of other molecules such as the amino acid sequence of the 12-residue membrane-translocating peptide (Ala-Ala-Val-Leu-Leu-Pro-Val-Leu-Leu-Ala-Ala-Pro) can be incorporated into this polyplex to either target, increase adsorption, internalization or nuclear localization of the polyplex. As used herein, however, the term "peptide" is intended to include mimetics and the term "amino acid" is intended to include D-form amino acids and modified amino acids.

Examples

Example 1

Method for synthesis of bile acid conjugates (BAC)

BAC is synthesized by solid phase chemistry on a peptide synthesizer. A six L-arginine peptide was first synthesized on the resin bed using standard 9-fluorenylmethoxycarbonyl (FMOC) chemistry. To attach the bile acid salt an excess of chendoxycholic acid was added to the resin and allowed to react with the immobilized peptide. After conjugation, BAC was cleaved from the resin and purified to greater than 95% purity by HPLC.

Example 2

Construction of Expression Plasmids

An expression plasmid using an optimized Cytomegalovirus (CMV) early promoter-enhancer to drive transgene expression (pCF1) was utilized. For cell localization and transfection expression experiments the firefly luciferase cDNA was used as a reporter transgene (pCF1-luc). Plasmid backbones which are chosen to contain the origin of replication and an antibiotic resistance gene or other selectable marker allowing the growth and maintenance of the plasmid in its bacterial host. Other attributes to facilitate the production of plasmid DNA are: 1) the ability of the plasmid to produce high copy number in the host bacteria; 2) a eukaryotic transcriptional promoter to initiate the synthesis of mRNA when it enters the target cells; 3) the DNA coding sequence encoding the protein of interest with a translational start codon (ATG) in the sequence for initiation of protein synthesis and a stop

codon to terminate translation; and 4) an optional transcription termination sequence.

Example 3

Preparation of BAC/pDNA Complexes

Typically an equal volume of BAC solution at 3 mg/ml (2.25 mM) was slowly added with mixing to a pDNA solution at a concentration of 250μg/ml to form a neutrally charged complex. Mass spec analysis has provided evidence that the major ionic species in the BAC has a charge of +3 per BAC molecule (MW = 1329) under neutral conditions (data not shown). Assuming a charge of –2 per base pair of the 4550 bp pDNA (MW = 3,003,000). (Total DNA charge = -9100), a net neutral charge would theoretically require a ratio of about 3033:1 (BAC:pDNA). Physical measurements of the BAC/pDNA complex appear require about 10-fold more BAC to neutralize the pDNA charge (see Table I). The presence of buffer salts bound to the BAC during synthesis and purification may partially explain this observation.

Table I

BAC amount μg/ml	BAC:pDNA molar ratio	Particle Charge determined by Zeta Potential
50	8070:1	Negative
200	32,280:1	Neutral
2000	322,799:1	Positive

Example 4

BAC condenses plasmid DNA.

The ability of BAC to condense DNA was determined using transmission electron microscopy (TEM). TEM of a BAC/pDNA particle complex is shown in Figure 1. The mole ratio of BAC:pDNA was 1581:1, which retained a net negative charge on the particle. The samples after mixing were diluted in water, and a drop applied to the carbon-coated formvar-film grid. These were dried, stained and observed in a TEM scope. Even at this low molar ratio of BAC:pDNA clear rod shaped particles (toroids) were formed.

Example 5

BAC condensed plasmid DNA forms discrete particles.

NICOMP particle size analysis is shown in Figure 2. 1 ml of 14 ug/ml DNA plasmid was mixed with 1 ml of BAC at the concentrations shown in Table 1. Also shown are the calculated molar ratios and particle charges.

Particle sizes were measured at a specific time after mixing of the pDNA with the BAC.

Figure 2 panels a-d show some representative results of these experiments. Mixtures of pDNA and BAC start forming particles immediately. However, with increasing time these particles appear to aggregate, so that at later times particles larger than 1 μm were observed.

Example 6

BAC condensed plasmid DNA particle size in simulated gastric or intestinal fluid.

The behavior of the pDNA/BAC complex in buffers with different pH's to simulate gastric fluid (SGF) or intestinal fluid (SIF). pDNA and BAC (BAC:pDNA: = 80,699:1, slightly positive charge) were mixed first in water and then mixed with the same volume of simulated gastric fluid (SGF, 0.1 M HCl, pH 1.2) or simulated intestinal fluid (SIF, 0.05 M phosphate buffer, pH 6.8). Measurements were made after two hours incubation at 37°C. After 2 hours, most of the DNA complexes in water (Figure 3a) and SIF (Figure 3b) were observed to be greater than 1 um in size. However, over 30% of DNA complex incubated in SGF (Figure 3c) were observed to be under 1 um size. These results indicate that pH has some effect on the physical behavior of BAC/pDNA complexes.

Example 7

Measurement of the zeta potential (net charge) of the particle complexes were made after mixing BAC/pDNA in water. Additional aliquots of BAC were added and the zeta potential remeasured (Figure 4).

Example 8

DNase protection assays - The ability for BAC complex formation to protect DNA from endonuclease activity was examined. We have utilized the ability of polyanions such as polyaspartic acid (PAA) to competitively dissociate the polycations from DNA (33).

1) The mixture of luciferase BAC/pDNA was stored at room temperature for 2.5 hours (BAC/pDNA: = 9038:1).

2) 10 µl of 10 mg/ml PAA or milli-Q water was added to each sample (5 µl of DNA complex mixture) and stored at room temperature for 2 hours.

3) The molar ratio was (DNA:BAC:PAA= 1:9038:13,572), the charge ratio (DNA:BAC:PAA=1:3:388), assuming BAC has 3 positive charges and PAA has 260 negative charges.

4) 5 ul of 100 kunit/ml DNaseI or milli-Q water was added to each sample and those samples were incubated at 37°C for 15 min and heated at 70°C for 15 min.

5) 2 ul of 500 mM EDTA and 1 ul of 5% SDS were added to each sample and heated at 55°C for 3 hours to dissociate the BAC from the pDNA. Samples were then electrophoresed in an agarose gel and stained with ethidium bromide (Figure 5).

Conclusions from DNAse protection assays: Extremely harsh conditions are required to dissociate BAC from the BAC/pDNA complex (1% SDS at 55°C for 2 hours, 1 hour was not enough to reach a plateau dissociation level, data not shown).

DNaseI together with BAC in the mixture altered the effect the dissociation by SDS (Lane 2). The DNA sample which was incubated at 37°C for 15 min with DNaseI showed almost complete fragmentation (Lane 3). The BAC/pDNA sample incubated in the presence of PAA and DNAseI showed

less intact DNA banding (Lane 1) than BAC/pDNA samples with or without treatment with PAA (Lanes 4 & 5 respectively).

These results show that DNA was protected by BAC from DNAseI activity.

Example 9

***In Vitro* Transfection Assays:** To examine the capability of the BAC/pDNA complexes to transfect cells *in vitro*, we examined the ability of BAC to transfect Hela tissue cultures using the firefly luciferase gene. In transient transfection assays (24 h), BAC enhanced the transfection efficiency in combination with liposomes (data not shown). But, more interesting was the ability of the BAC/pDNA to transfect Hela cells alone as a single agent (Figure 6). The charge ratio was approximately neutral. This experiment has not been optimized. The approximate molar ratios are indicated in parenthesis.

Example 10

***In Situ* Intestinal Absorption:** In order to examine the capability of the BAC/pDNA complex to bind and/or be absorbed onto the intestinal lumen cells we tested the absorption of nicked-radiolabeled pDNA in a single pass perfusion experiment. Using isolated ileal or jejunal segments radio-labeled BAC/pDNA mixtures mixture was mixed with HEPES buffer, pH 7.4, and perfused at jejunal and ileal sites. After 30 min of steady state, sample fractions were collected and the absorbed fraction of BAC/pDNA calculated. BAC/pDNA mixture used were (pDNA:BAC= 1:0 negatively charged, 1:27,000 neutrally charged, 1:54,000 positively charged).

Between 20-40% of the "neutrally charged" BAC/pDNA complex was absorbed in a single pass of the intestine (Figure 7). If true, then we would expect that this complex should be completely absorbed after oral dosing. The mechanism of this absorption, whether by binding to specific transporters such as the ileal bile acid transporter or by a non-specific mechanism remains to be determined.

Patent applications and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These applications and publications are incorporated herein by reference to the same extent as if each individual application or publication was specifically and individually incorporated herein by reference.

The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims, including all equivalents thereof, are intended to define the scope of the invention.

What is claimed is:

Claims

1. A method for increasing the blood levels of secretable therapeutic proteins in mammals, the method comprising: introducing a formulation into a target cell, the formulation comprising a DNA construct not packaged in a viral particle, wherein the construct encodes a functionally active protein polypeptide that mediates increasing the protein levels in the bloodstream.

2. The method of claim 1, wherein said introducing is by oral administration.

3. The method of claim 1, wherein functionally active protein is delivered to the bloodstream for a period of from about two to four days.

4. The method of claim 1, wherein the target cell is a gastrointestinal cell other than an intestinal stem cell.

5. The method of claim 5, wherein the gastrointestinal cell is within the small intestine.

6. The method of claim 5, wherein the gastrointestinal cell is a cell within the large intestine.

7. The method of claim 1, wherein said introducing is by parenteral administration.

Appendix

Table 2

Exemplary Proteins for Gene Therapy

Insulin	interferon-alpha.2B	human growth hormone (hGH)
transforming growth factor (TGF)	erythropoietin (EPO)	ciliary neurite transforming factor (CNTF)
clotting factor VIII	insulin-like growth factor-1 (IGF-1)	bovine growth hormone (BGH)
granulocyte macrophage colony stimulating factor (GM-CSF)	platelet derived growth factor (PDGF)	interferon-alpha.2A
clotting factor VIII	brain-derived neurite factor (BDNF)	thrombopoietin (TPO)
insulintropin	tissue plasminogen activator (tPA)	
IL-1	IL-2	urokinase
IL-1 RA	streptokinase	superoxide dismutase (SOD)
adenosine deamidase	catalase	calcitonin
arginase	fibroblast growth factor (FGF) (acidic or basic)	neurite growth factor (NGF)
phenylalanine ammonia lyase	granulocyte colony stimulating .gamma.-interferon factor (G-CSF)	L-asparaginase
pepsin	uricase	trypsin
chymotrypsin	elastase	carboxypeptidase
lactase	sucrase	intrinsic factor parathyroid hormone (PTH)-like hormone
calcitonin	Ob gene product	cholecystokinin (CCK)
glucagon	glucagon-like-peptide I (GLP-1)	insulinotrophic hormone

Table 3

Exemplary Classes of Proteins for Gene Therapy

proteases	pituitary hormones	protease inhibitors
growth factors	cytokines	somatomedians
chemokines	immunoglobulins	gonadotrophins
interleukins	chemotactins	interferons
lipid-binding proteins		

Various disease conditions are amenable to treatment using the gene therapy of the invention. One skilled in the art can recognize the appropriate protein that should be produced by the invention for treating specific disease conditions. Exemplary diseases that are amenable to treatment using the subject invention, and exemplary, appropriate proteins which can be used in treating these diseases, are shown in Table 3.

Table 4

Exemplary Disease Conditions or Deficiencies Amenable to Gene Therapy

Enzyme Deficiency	Erythropoietin	Catalase
Endotoxic Shock/Sepsis	Adenosine deaminase (For treatment of severe combined immunodeficiency)	Lipid-binding protein (LBP)
Purine nucleotide phosphorylase	Galactosidase	beta-glucuronidase
Antioxidants for Cancer	Therapy Anemia	Superoxide dismutase
Cancer		Growth Factors (for use in wound healing, induction of red blood cell formation, etc.)
alpha.-Interferon	beta.-Interferon	Epidermal growth factor
Granulocyte colony stimulating factor (G-CSF)	alpha-IL1	gamma-Interferon
Phenylalanine ammonia lyase	Transforming growth factor	Arginase
Erythropoietin	L-asparaginase	Thrombopoietin
Uricase	Insulin-like growth factor-1	Insulin
Human growth hormone	Monoclonal antibodies	Tissue necrosis factor
Cardiovascular Disease	Diabetes	Tissue plasminogen activator

Urokinase (native or chimeric)	Glucagon	alpha..sub.1 -antitrypsin
Insulinotrophic hormone	Clotting disorders	Antithrombin-III
Other proteases or protease inhibitors	Clotting factor VIII	
Apolipoproteins (particularly B-48)	Circulating Scavenger Receptor	APO A1.(Converts low-density lipoproteins to high-density lipoproteins)
Gastrointestinal and Pancreatic Deficiencies	Obesity and Feeding	
Pepsin (for esophageal reflux)	Ob gene product	Cholecystokinin (CCK)
Trypsin	Chymotrypsin	
Bone diseases		
Elastase	Carboxypeptidase	Calcitonin
Lactase (for lactose deficiency)	PTH-like hormone	Sucrase
Intrinsic Factor (pernicious anemia)	Myasthenia gravis (acetylcholine receptors)	Graves' disease (thyroid-stimulating hormone receptor)
Organ-Specific Autoimmune diseases (target of antibody in parentheses)	Thyroiditis (thyroid, peroxidase)	Insulin-resistant diabetes with acanthosis nigricans or with ataxia telangiectasia (Insulin receptor)
Allergic rhinitis, asthma (Beta.sub.2 -adrenergic receptors)	Juvenile insulin-dependent diabetes (insulin, GAD65)	Pernicious anemia (gastric parietal cells, vitamin B.sub.12 binding site of intrinsic factor)
Addison's disease (adrenal cells)	Idiopathic hypoparathyroidism (parathyroid cells)	Spontaneous infertility (sperm)
Premature ovarian failure (interstitial cells, corpus luteum cells)	Pemphigus (intercellular substance of skin and mucosa)	Bullous pemphigoid (basement membrane zone of skin and mucosa)
Primary biliary cirrhosis (mitochondria)	Autoimmune hemolytic anemia (erythrocytes)	
Idiopathic thrombocytopenic purpura (platelet)	Idiopathic neutropenia (neutrophils)	Vitiligo (melanocytes)
Osteosclerosis and Meniere's disease (type II collagen)	Chronic active hepatitis (nuclei of hepatocytes)	Systemic Autoimmune Diseases (defect/organ affected in parentheses)
Goodpasture's syndrome (basement membranes)	Rheumatoid arthritis (.gamma.-globulin, EBV-related antigens, collagen types II and III)	Sjogren's syndrome (.gamma.-globulin, SS-A (Ro), SS-B (La)

Systemic lupus erythematosus (nuclei, double-stranded DNA, single-stranded DNA, Sm ribonucleoprotein, lymphocytes, erythrocytes, neurons, gamma-globulin)	Scleroderm (nuclei, Scl-70, SS-A (Ro), SS-B (La), centromere	Polymyositis (nuclei, Jo-1, PL-7, histadyl-tRNA synthetase, threonyl-tRNA synthetase, PM-1, Mi-2
Rheumatic fever (myocardium heart valves, choroid plexus)		

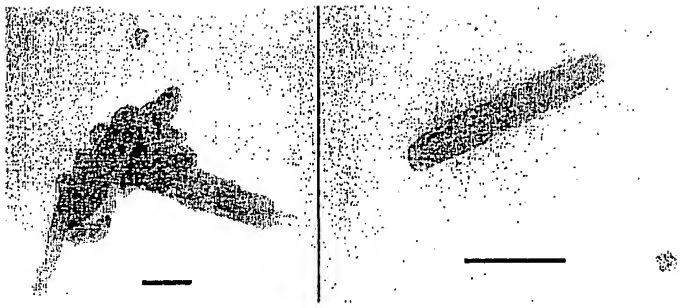


Figure 1. TEM of BAC/pDNA complex. A negatively charged mixture of BAC/pDNA was applied to a carbon-coated formvar-film grid and allowed to dry. The samples were stained with uranyl acetate before viewing. Bars are 100 nM long.

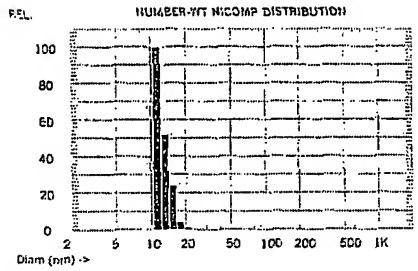


Figure 2a – Particle sizing of pDNA only

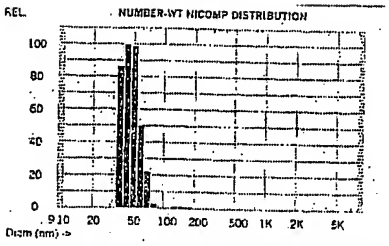


Figure 2b - pDNA + 50ug/ml BAC. Time until measurement after mixing 0 min.
(complex is negatively charged)

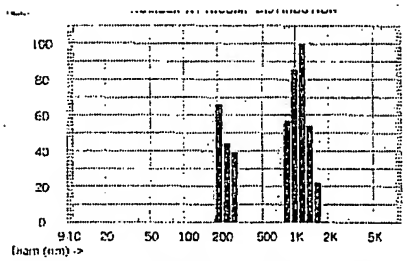


Figure 2c - pDNA + 200ug/ml BAC. Time until measurement after mixing 0 min.
(complex is neutrally charged)

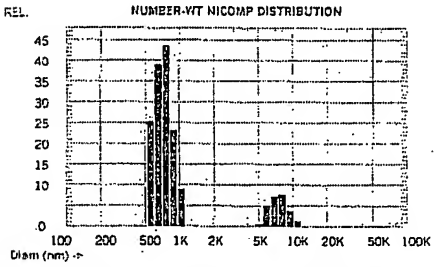


Figure 2d - pDNA + 200ug/ml BAC. Time until measurement after mixing 2 hours.
(complex is neutrally charged)

Figure 2. Measurement of BAC / plasmid DNA particle formation

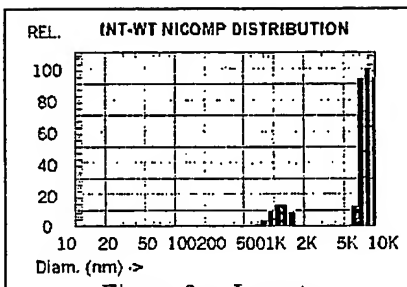


Figure 3. BAC condensed plasmid DNA particle size in simulated gastric or intestinal fluid

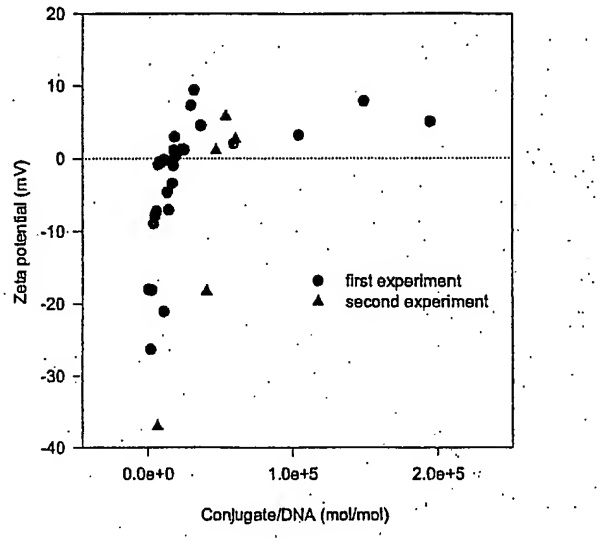
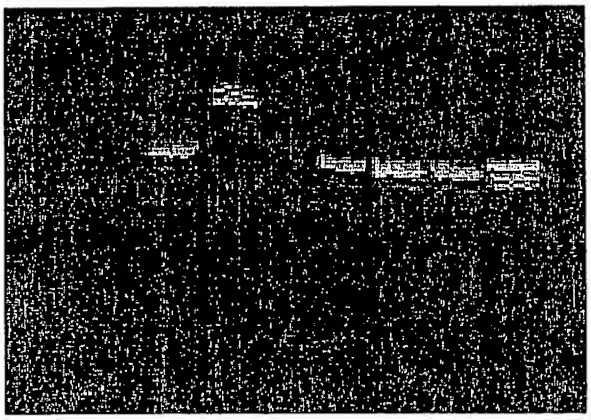


Figure 4. BAC/pDNA Particle Zeta Potential Measurements
800 μl of 11.07 μg/ml DNA plasmid was mixed with 800 μl of 50 μg/ml BAC (DNA:BAC = 1:10,205 -negatively charged).

10 μl of 500 μg/ml or 1 mg/ml BAC was added and zeta potential was remeasured in experiment 1 (•). 200 μl of 10 μg/ml pDNA was prepared and 2 μl of 3 mg/ml BAC was added before zeta potential remeasurement in experiment 2 (π).

Figure 5. Endonuclease Protection Assay

Lanes 1 2 3 4 5 6 7



Lanes
1) DNA + BAC + PAA + DNaseI
2) DNA + BAC + DNaseI
3) DNA + DNaseI
4) DNA + BAC + PAA
5) DNA + BAC
6) DNA
7) DNA size standard

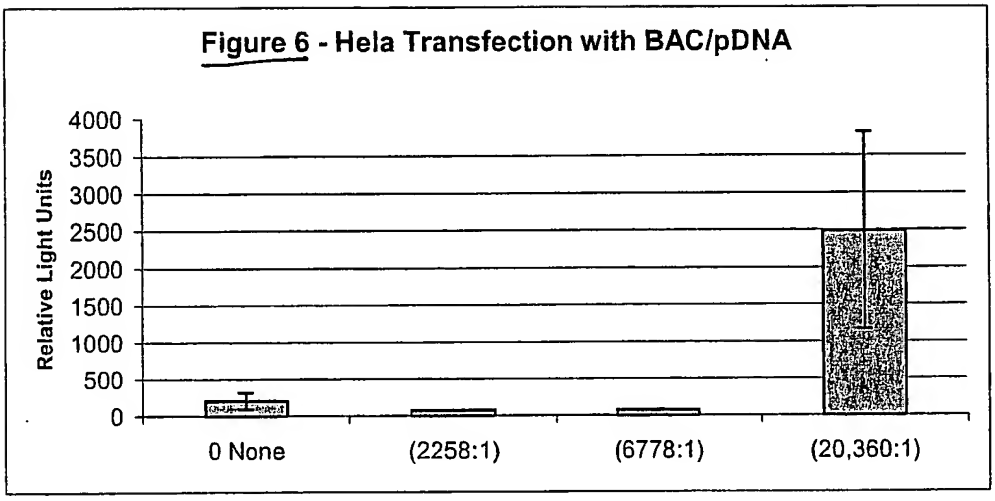


Figure 6. ***In vitro*** **transfection of Hela cells with BAC/luciferase pDNA.**

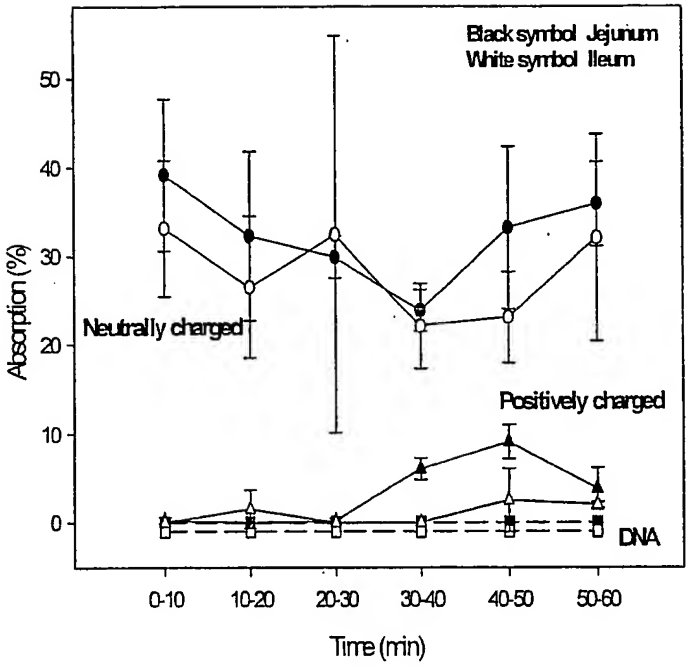


Figure 7. ***In situ*** **perfusion of radiolabeled BAC/pDNA in intestinal segments.**